The University of Chicago

THE CROSS-IMMUNE RELATIONSHIP OF VARIOUS STRAINS OF PLASMODIUM CATHEMERIUM AND P. RELICTUM

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

1937

By

W. B. REDMOND

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THE CROSS-IMMUNE RELATIONSHIP OF VARIOUS STRAINS OF PLASMODIUM CATHEMERIUM AND P. RELICTUM

W. B. REDMOND

From the Department of Bacteriology and Parasitology, University of Chicago

Although the cross-immune reactions of numerous species and strains of avain malaria have been studied by various workers, the present investigation is an attempt to clarify some of the problems associated with the varying reciprocal cross infections that have been observed. The two new strains which have been used vary in some characteristics from the classical strains of the corresponding species used by most investigators. Therefore, an attempt has been made to correlate the immune reactions to them with other variations.

HISTORICAL

Since the recognition of the existence of more than one type of avian malaria and their separation into definite species by Hartman[1] repeated attempts have been made to differentiate species by their immunological variations. In discussing the two species described by him, Hartman states: "Birds which had had an infection of one species were as easy to infect with either or both the other species as were birds which had never been infected." Manwell[2] using two of Hartman's species observed that neither gave any immunity to the other species when inoculated into birds with latent infections. The Sergent brothers and Catanei[3] found that birds having latent infections of Plasmodium relictum developed variable infections when inoculated with P. rouxi. Likewise, they[4] observed that birds with latent infections of P. cathemerium developed only subacute infections when inoculated with P. relictum. When inoculations of P. cathemerium were made into 5 birds carrying latent infections of P. relictum one acute infection and 4 subacute infections resulted. P. cathemerium produced 2 fatal attacks when inoculated into birds with latent infections of P. rouxi, but another bird showed no increase in number of parasites. Kikuth[5] was unable to demonstrate any cross-immunity between P. elongatum and P. cathemerium, P. inconstans (relictum) or P. circumflexum. Gingrich[6] established the immune relationship of P. cathemerium, P. relictum, P. elongatum, and P. rouxi as follows:

1. A latent P. cathemerium infection produced a partial cross-immunity to P. relictum and P. rouxi, but no cross-immunity to P. elongatum.

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The author is particularly indebted to Dr Clay G. Huff under whose supervision the work was done, and to Dr. G. Robert Coatney for strain M of P. cathemerium.

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1. Arch. f. Protistenk. **60:** 1, 1927.
2. Am. J. Hyg. **9:** 308, 1929.
3. Arch. Inst. Pasteur d'Algerie **7:** 165, 1929.
4. Bull. Soc. path. exot. **24:** 327, 1931.
5. Zentralbl. f. Bakt. (Abt. 1) **121:** 401, 1931.
6. J. Prev. Med. **6:** 197, 1932.

2. A latent P. relictum infection resulted in complete cross-immunity to P. cathemerium, but no cross-immunity to P. rouxi or P. elongatum.

3. A latent infection of P. elongatum conferred no cross-immunity to any of the other 3 species.

4. A latent or chronic infection of P. rouxi established a partial cross-immunity to P. cathemerium, but none to either P. relictum or P. elongatum. Manwell[7] found no cross-immunity between P. nucleophilum and P. cathemerium, P. circumflexum, P. elongatum, or P. rouxi. A latent infection of P. relictum produced a slight decrease in a subsequent infection of P. nucleophilum, but reverse injections showed no cross-immunity.

From these results one can readily see there is no clear-cut differentiation of species possible on the immunological bases by tests so far employed. Both Kikuth[5] and Manwell[8] state that immunological differentiation is not sufficient and that other criteria, especially morphological, are necessary for establishing species. Gingrich,[6] in an attempt to explain the varying cross-immune reactions of P. cathemerium, P. relictum and P. rouxi, believed the immunity to be specific but that there are one or more antigenic factors common to each species of this group which account for the partial cross-immunity among them. From the nature of the variations a group reaction does not seem to be an adequate explanation. Studies on other new strains, morphologically identical with two of these species but varying in physiological and immunological characteristics have made it possible to offer an explanation based on the relative virulence of the various strains.

MATERIAL AND METHODS

Two strains of P. relictum and 3 of P. cathemerium, have been used in these experiments

Strain W: The "Whitmore" strain of P. relictum was isolated by Whitmore in 1913, and has been used by numerous workers. For a full description of it see Gingrich.[6]

Strain R: This strain, morphologically identical with P. relictum, was isolated by Huff[9] from a robin secured from Kansas, Illinois and shown by him to possess a strict quotidian periodicity with segmentation taking place about 7 A.M.

Strain N: This is a single-cell strain which was isolated by Dr. Stauber from the original Hartman strain of cathemerium. It has been used extensively in this laboratory for several years and exhibits all the characteristics of the original P. cathemerium strain.

Strain A: Morphologically this strain is identical with Strain N of P. cathemerium. It was isolated from a grackle in 1933 by Dr. Clay G. Huff, and is similar in all respects to that of strains H and N, except in virulence. Of 20 birds infected with this strain 14, or 70%, died of the infection. The peak of the infection is generally high, frequently reaching one or more parasites per red blood cell.

Strain M: This strain was isolated from a magpie in Nebraska by Coatney and Roudabush[10] and was supplied me by Dr. G. Robert Coatney. It is very virulent, is morphologically identical with the original P. cathemerium strain, and is apparently identical with strain A.

These strains and many others have been maintained in canaries in the laboratory for varying lengths of time and little, if any variation has been observed even in the oldest ones. In these experiments female canaries were used as the vertebrate host. The inoculations were made by intravenous injections in the great majority of cases. Intramuscular and intraperitoneal inoculations were made in a few cases. Infected blood from the toe or jugular vein was

7. Proc. Soc. Exper. Biol. & Med. **32:** 391, 1934.
8. Am. J. Trop. Med. **15:** 265, 1935.
9. J. Parasitol. **23:** 400, 1937.
10. Am. Midland Naturalist **18:** 1005, 1937.

collected in an equal amount of heparinized physiological normal saline solution (0.85%). When the blood was to be taken from the jugular vein the bird was etherized, the feathers were removed from the side of the head and neck, the vein was cut, and all the blood was allowed to escape into a dish containing the saline solution. Intravenous injections were made into the median vein of the middle toe and intramuscular injections into the breast muscles by means of a 27 gauge needle. Sterile apparatus was used as far as possible. The skin of the bird was cleaned with alcohol each time blood was secured or injections made.

Blood smears for examination were made regularly between 3 and 4 o'clock each afternoon. After drying, they were fixed from 1 to 10 minutes in absolute methyl alcohol and stained with Gingrich's modification of MacNeal's tetrachrome stain. Smears were made daily from all superinfected birds as long as any parasites were found. In infections having 50 parasites or less per 10,000 red cells the records show only estimates in all but a few instances. The observed values for infections above 150 parasites per 10,000 red cells have been calculated to have a probable error of 10% or less; those for infections between 50 and 150 parasites per 10,000 red blood cells a probable error of 15% or less. The probable error for any observed values given for infections below 50 parasites per 10,000 red blood cells has been calculated to be 20% or less. Where no figures are given as observed values of the infection, symbols are used to indicate the range within certain limits. The variations in these estimated values are not large enough to be of very great significance. In most cases they are used where the infection is either just beginning to rise, or is disappearing. The symbols employed by Gingrich are used in order to facilitate comparison with his results. Explanations of these symbols are given with the tables. Slides recorded as negative were examined for 10 minutes or more and if no parasites were seen no counts of red cells were made. Examinations were made just prior to superinfection to determine if the infections of the experimental birds were latent. All birds were kept in mosquito-proof cages in order to prevent chance mixing of the strains by mosquitoes.

NORMAL INFECTIONS

Strain R (P. relictum var. matutinum).—A comparison of the physiological characteristics of the strains studied soon reveals the fact that they are not correlated with the morphological characteristics. Though morphologically strain R is a variety of P. relictum it does not resemble this species otherwise. The period of the acute infection lasts about 5 or 6 days. The number of parasites increases rapidly for about 3 days, normally reaching a peak of infection of 1,000 to 1,200 per 10,000 red cells, after which a sudden decrease occurs. Intravenous injections made with blood so heavily infected that numerous parasites could be found in smears made immediately after the injection did not cause any appreciable increase in the number of parasites in the subsequent infections. A bird having a relatively high infection was killed and all the infected blood was injected into 2 normal birds. On the third day following the bird having the highest infection was killed and all the blood was injected into 2 normal birds. This was continued for about 2 weeks, but the highest infection obtained was only 1,660 parasites per 10,000 red blood cells. Two or 3 such transfers of strain N or W usually resulted in very heavy infections, generally killing the bird. In this manner it is possible to increase the number of parasites in the 2 latter strains to equal that of the red blood cells. After the sixth day of the infection with strain R few, if any, parasites can be found on examination of blood smears. No relapses have occurred except in 2 birds in which there was an intercurrent bacterial infection. Occasionally a bird carrying this strain dies, but it is difficult to say that the infection was the cause of death, because in working with a large number of birds it has been observed that about as high a percentage of those that are uninfected die as of those that are infected with strain R.

Strain A and strain M of P. cathemerium.—These 2 strains, though isolated in different parts of the country and from different hosts, seem to be identical and are morphologically identical with strains H or N, of P. cathemerium. Since no distinct difference has been found between them they will be considered together. The most outstanding characteristic of both these strains is their virulence. Seventy percent of the birds infected with strain A and 70 to 80 percent of those infected with strain M die of the infection. The parasites continue to increase in the blood until almost every erythrocyte contains one or more parasites. The infection seldom reaches a crisis before the bird dies. When the bird survives, the infection lasts from 2 to 3 weeks with parasite counts ranging from 5,000 to 6,000 per 10,000 red cells. Even in birds injected with so few parasites that none could be found until the second day the parasites continued to increase until the bird died.

Comparison with the N (P. cathemerium) and W (P. relictum) strains.—These 2 strains have been used so extensively by numerous workers that it is unnecessary to go into detail in describing them. Examination of the literature dealing with these strains shows that the normal infections appear to run about the same courses. The normal infection of strain N (P. cathemerium) reaches a crisis on or about the sixth day after the parasites appear, with a maximum number of parasites between 2,500 and 4,000 per 10,000 red blood cells. The infection of strain W (P. relictum) has a tendency to last longer, reaching a crisis on or about the eighth day with counts amounting to 4,000 or more per 10,000 red cells. No single infection can be considered to be typical of either strain, however, as there is considerable overlapping. Occasionally an infection of strain N will continue to rise for 8 or 10 days. The number crisis, too, may exceed that of the average W strain. Comparisons of numerous infections of the two strains demonstrate that the latter strain runs a somewhat longer course than strain N. After the crisis of an infection of strain W parasites are found to be rather numerous, sometimes for as long as 2 weeks. Gingrich states that in latent or chronic infections of this strain 50% of the birds show an occasional parasite on examination of smears.

Figure 1 shows comparative curves of daily number counts of infections of the 4 strains just described (considering strains A and M to be identical). Strains R and A stand out distinctly as having the lightest and heaviest infections, respectively. The curve for strain R may be slightly higher at the peak than normal as the birds from which these counts were made were part of the experimental attempt to increase the height of the infections of this strain. Each curve of this chart is compiled from two or more infections by superimposing the individual curves on each other so that the peaks fall on the same day. Although a more prominent number crisis is indicated in this way the resulting curve is considered to be more typical than one having 2 or 3 peaks, or no peak at all. It is significant that the parasites of each of the 4 strains increase at about the same rate for the first 3 days. At this time infections with strain R begin to decline sharply while the others continue to rise. Mixed infections of strains R and N inoculated simultaneously show the same relationship. Strain R reaches a crisis on the third day and begins to

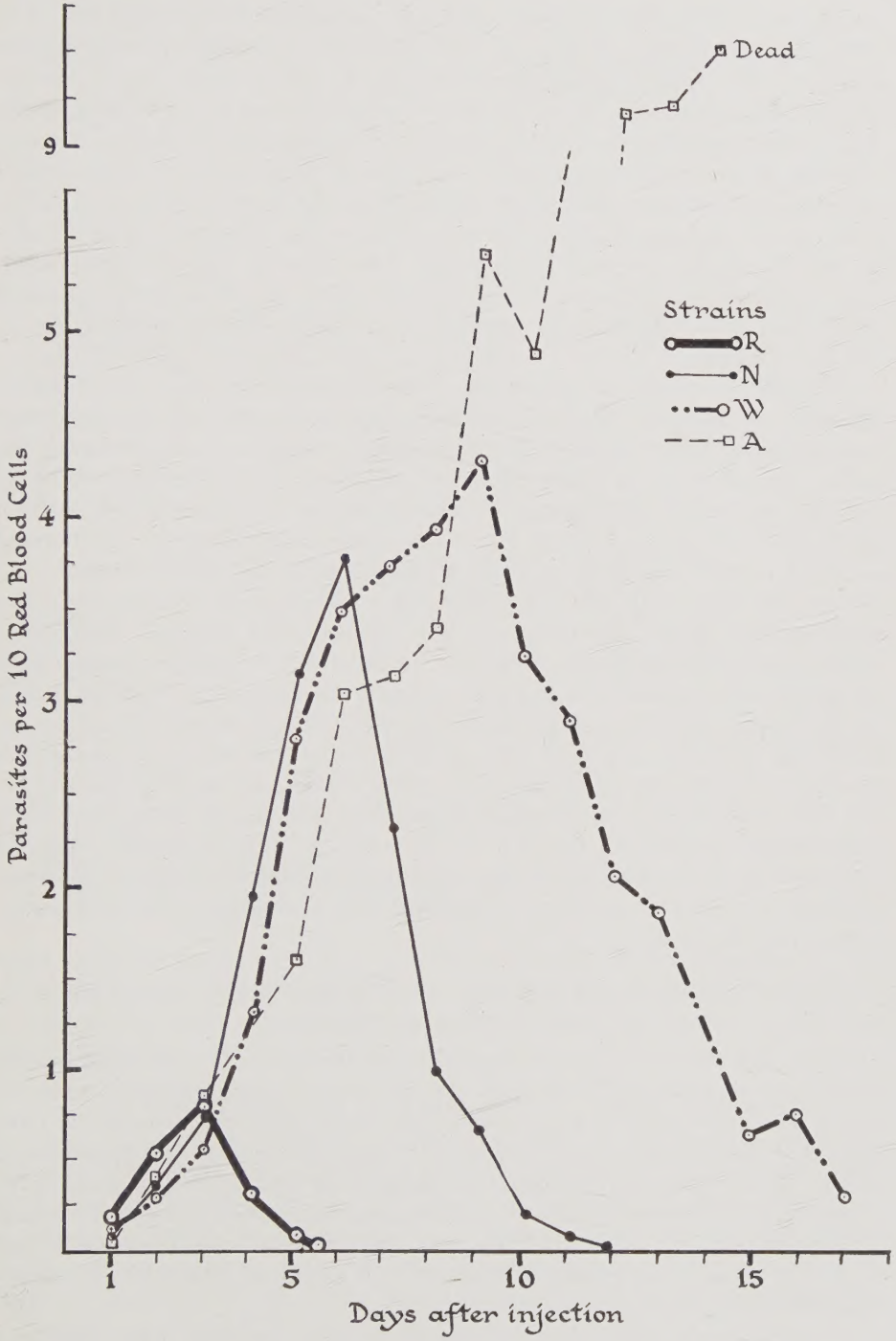

Fig. 1.—Comparison of normal infections of strains N and A of P. cathemerium and strains R and W of P. relictum. Each curve is a composite of two or more infections.

decrease while the N infections continue to rise until the sixth day. The differentiation of the 2 strains in the same infection is made possible by the fact that the respective segmentation periods are 12 hours apart. Smears were made at 4:00 P.M. at which time the parasites of strain N were in the segmenting stage just before liberation of the merozoites, while the parasites of strain R were still very small. The curve of strain A is compiled from 2 infections which were both fatal. In one bird the number increased to 1.1 parasites per red cell. A slight difference between the infections of the strains N and W can be observed in the graph. The crisis in strain N occurs from 1 to 2 days previous to that of strain W and after the crisis, the parasites of the former strain decrease in number and disappear more rapidly than in the latter strain.

To obtain a numerical comparison of the normal infections of these heterologous strains shown in figure 1 the surface plane of each curve as reproduced was measured with a planimeter. The relative values are as follows for the different strains: strain R .99; strain N 7.88; strain W 17.43; strain A 28.41. In relation to strain R (taken as 1), the values are as follows: strain N 7.96; strain W 17.60; strain A 28.69. It must be remembered that the infections of strain A reached no crisis and had no period of decline comparable to the others. The planimeter reading of this strain was obtained by drawing a line perpendicular from the abscissa to the highest point reached. This point corresponds with the death of the bird. In order for the values of strain A to be comparable to those for the other strains the planimeter reading should be multiplied by two, giving a value of 56.82. This is nearer the actual relationship of the virulence of these strains. If the sum of the daily parasite counts per 10,000 red blood cells to the peak is taken for each strain, and using that of strain R as 1, a similar relationship is obtained. The figures vary somewhat as follows: for strain R, 1; for strain N, 9; for strain W, 14.6 and for strain A, 30. In other words up to the peak of infection there are 30 times as many parasites in the blood of birds infected with strain A as in those infected with strain R.

CROSS INFECTIONS OF THE STRAIN R (*P. relictum* var. *matutinum*) WITH STRAINS N AND W

Although strain R is morphologically identical with the various strains of *P. relictum*, it has a strict quotidian asexual cycle with segmentation at about 7 A.M. The cycle resembles that of known strains of *P. cathemerium* in having a 24-hour periodicity, but differs by 12 hours in the time of segmentation. Strain R then has morphological characteristics identical with those of *P. relictum*, but has physiological manifestations resembling those of *P. cathemerium*. Since there is considerable cross-immunity between *P. cathemerium* and *P. relictum* it was thought that strain R might possibly show an intermediate step in the cross-immunity series.

In contrast to what Gingrich[6] and others have found in other strains of *P. relictum* strain R does not produce an effective immunity to *P. catemerium* or to strain W of *P. relictum*. Tables 1 and 2 show the results of cross infections of strain R with strains N and W. Parasites are found for

TABLE 1.—*Results of Inoculating Strain R into Birds with Latent Infections of Various Strains*

Recipients: Bird No.	Type of Latent Infection	Days*	Strain Inj.	Parasites per 10,000 Red Blood Cells: Days after injection #	1	2	3	4	5	6	7	8	9	10	11	12	13
4	N	237	R	57	†	0	0		0								
5	N	116	R	71	+	+	0	0	0								
6	N	230	R	46	+	†	†	0	0	0	0	0					
10	R		R	33	†	0	0	0									
11	R		R	47	†	0	0	0									
12	R		R	39	†	0	0										
22	W	23	R	37	†	†	0	0	0	0	0	0	0				
23	W	97	R	29	†	†	0	0	0								
24	W	99	R	26	†	†	0	0	0								

* Number of days since original infection.
\# Number of parasites 10 minutes after injection.
† Estimated number of parasites 10 or less per 10,000 red cells.
+ Estimated number of parasites 10 to 35 per 10,000 red cells.

several days after intravenous inoculations of either the N or W strain on birds carrying latent infections of strain R. In most cases an actual increase in number of parasites occurs (table 2), a condition called partial cross-immunity by Gingrich. When parasites of strain R (table 1) are injected into

TABLE 2.—*Results of Inoculating Strain W and Strain N into Birds Carrying Latent Infections of Strain R*

Recipients: Bird No.	Type of Latent Infection	Days*	Strain Inj.	Parasites per 10,000 Red Blood Cells: Days after injection #	1	2	3	4	5	6	7	8	9	10	11
13	R	12	W	19	67	78	117	159	179	217	110	68	+	†	0
14	R	8	W	64	113	73	194	261	389	466	245	213	256	348	327
15	R	130	W	77	58	†	†	47	93	141	257	286	384	163	88
16	R	32	N	63	96	139	57	†	0	0		0			
17	R	24	N	52	133	215	197	†	†	0	0				
18	R	20	N	46	94	231	151	†	†	0	0	0			

* Number of days since original infection.
\# Number of parasites 10 minutes after injection.
† Estimated number of parasites 10 or less per 10,000 red cells.
+ Estimated number of parasites 10 to 35 per 10,000 red cells.

birds carrying latent infections of strains N or W they are removed immediately, showing that an effective complete immunity exists. Strain R of P. relictum is then, morphologically identical with the original strain, but does not produce a comparable immunity.

CROSS INFECTIONS OF STRAIN A OF P. CATHEMERIUM

With strain R.—Because of the virulence of strain A it has been difficult to obtain sufficient birds with latent infections for extensive cross infections. Nevertheless, enough to indicate its immune relationship to the other strains have been available. Birds with latent R infections showed only partial immunity when injected with parasites of strain A (see table 3). In view of previous work one would expect the P. relictum strain to produce complete cross-immunity to the P. cathemerium strain.

With strain N.—A more effective immunity to strain A is produced by strain N, however, than is produced by strain R (table 3). When strain A is inoculated intravenously into birds with latent N infections only a slight

increase in number of parasites results and all of the parasites disappear by the fourth or fifth day. Birds carrying latent infections of the strain H were also injected with this strain with the same results. Strains N and H do not produce complete immunity to strain A.

TABLE 3.—*Results of Inoculating Strain A of P. Cathemerium into Birds Carrying Latent Infections of Various Strains*

Recipients: Bird No.	Type of Latent Infection	Days*	Strain Inj.	Parasites per 10,000 Red Blood Cells: Days after injection #	1	2	3	4	5	6	7	8	9	10
7	N	62	A	160	388	237	44	0						
8	N	71	A	279	214	28	†	†						
9	N	71	A	136	223	64	†	0						
19	R	68	A	69	128	613	561	925	311	284	492	324	192	78
20	R	66	A	93	146	294	308	562	634	505	173	87	+	†
21	R	56	A	48	219	573	414	238	511	1275	1536	1082	784	636
25	W	190	A	198	286	351	212	+	0					
26	W	148	A	83	204	279	388	297	576	436	406	353	D	
27	W	133	A	67	116	234	327	351	215	308	254	183	121	66

* Number of days since original infection.
D Dead.
Number of parasites 10 minutes after injection.
† Estimated number of parasites 10 or less per 10,000 red cells.
+ Estimated number of parasites 10 to 35 per 10,000 red cells.

With strain W.—The results of cross infections of strain A with strain W have shown further that the former is a new and distinct strain of P. cathemerium. Table 3 shows the results of intravenous injections of strain A into

TABLE 4.—*Results of Inoculating Birds Carrying Latent Infections of Strain A with Strain W*

Recipients: Bird No.	Type of Latent Infection	Days*	Strain Inj.	Parasites per 10,000 Red Blood Cells: Days after injection #	1	2	3	4	5	6	7	8	9	10
1	A	1045	W	62	†	†	†	†	0					
2	A	126	W	47	+	0		0	0					
3	A	62	W	56	†	†	†	†	0	0				

* Number of days since original infections.
# Number of parasites 10 minutes after injection.
† Estimated number of parasites 10 or less per 10,000 red cells.
+ Estimated number of parasites 10 to 35 per 10,000 red cells.

birds carrying latent W infections. The lack of complete immunity is demonstrated by the increase in the number of parasites for 3 to 6 days. Just as outstanding also are the results (table 4) of inoculating birds carrying latent A infections with parasites of strain W. The quick removal of the parasites from the blood stream indicates an effective immunity to P. relictum not previously produced by any strain. The immune relationship of P. cathemerium and P. relictum as shown by Gingrich are reversed when strain A of P. cathemerium is employed in experiments instead of strain H, which he used.

MULTIPLE SUPERINFECTIONS AND ENHANCED RESISTANCE

The previous experiments have demonstrated that the strains of Plasmodium, at least in these 2 closely related groups, which run the longest

and highest course of infection produce the most effective cross-immunity In order to demonstrate just what part the increased number of parasites plays, large numbers of parasites were injected every few days into birds with latent infections, the object being to build up the immunity of the superinfected strain to the degree of effectiveness of the strain or strains to which it did not normally produce a complete immunity.

TABLE 5.—*Results of Injecting Strain N into Birds Previously Injected Repeatedly with Strain R*

Bird No.	No. of Superinj.	Test Strain	Parasites per 10,000 Red Cells										
			Days after injection										
			1	2	4	5	6	7	8	10	12	14	15
36	4	N	†	†	+	+	0	†	0	0			0
37	6	N	0			0	0	0	0	+	†	0	
38	2	N	0			†	+	95	93	0			
39	3	N	0	0		0	†	+	54	†		0	
40	2	N	0				0		†	62	178	62	†
41	4	N	0	0	0								

† Estimated number of parasites 10 or less per 10,000 red cells.
+ Estimated number of parasites 10 to 35 per 10,000 red cells.

The first series of superinfections were made on 7 birds carrying latent infections of strain R. All the blood from an infected bird, secured by bleeding from the jugular vein, was injected intravenously into 1 normal bird and 1 or 2 of the experimental birds, the former to serve for the next blood transfer. When the infection was heavy the blood was injected into 2 of the experimental birds, but if few parasites were present only one experimental bird was injected. Two factors governed the frequency of the superinfections. Birds were not injected until after the parasites of the previous superinfection could no longer be found on examination of blood smears. The other, and most important of the governing factors was the rapidity with which heavily infected birds could be secured. Since strain R naturally produces only a low grade infection in comparison with the other strains it was difficult to get a very heavily infected inoculum. This is offered as a possible explanation of the apparent failure of the attempt in this experiment to increase the resistance of the birds to cross infections of strain N, (table 5), since a later attempt was successful. The various birds received from 2 to 6 superinfections. Except for one bird (no. 34) a slight increase in number of parasites resulted when injections were made with strain N. The 2 birds receiving only 2 injections showed the greatest rise. One bird (37) which was given 6 superinfections showed the least increase in number of parasites after injection of strain N. This fact indicates that a greater number of superinfections probably would have increased the immunity to the point where it would be as effective against the injection of parasites of strain N as an immunity established by a primary infection of strain N.

The same birds were further superinfected with strain N. Since this strain produces a higher infection normally, than strain R it is not difficult to obtain heavily infected blood for injection. The same procedure as used in the R superinfections was followed. After the birds had been superinfected from 2 to 6 times, the immunity was tested with parasites of strain W, (table 6).

Again not all birds showed an increased resistance to the test strain. One bird (28) was given the test injection of strain W 9 days after the last superinfection. All others were tested after periods ranging from 37 to 52 days had elapsed. The complete removal of the test parasites in bird 28 and the apparent lack of removal in all but one of those tested later indicates that the enhanced resistance due to the superinfections may be lost after a few weeks.

TABLE 6.—*Results of Injecting Strain W into Birds Previously Injected Repeatedly with Strains R and N*

Bird No.	No. of Superinj.	Test Strain	Parasites per 10,000 Red Cells											
			Days after injection											
			#	1	2	3	4	5	6	7	8	9	11	12
28	4	W	225	54	0	0								
29	0	W	172	205	253	209	56	37	29	+	†	†	0	0
36	10	W	86	146	183	90	38	32	+	+	30		†	0
37	9	W	23	77	60	63	38	+	†	0	0	0		
38	6	W	72	51	127	148	181	98	176	38	†	0		
39	7	W	111	68	71	116	45	0	0	0				
40	6	W	154	19	†	0	0	0	0					
41	10	W	84	89	40	†	†	0	0	0				

10 minutes after injection.
† Estimated number of parasites 10 or less per 10,000 red cells.
+ Estimated number of parasites 10 to 35 per 10,000 red cells.

In bird 40 the test parasites were removed as readily as in bird 28. The enhanced resistance of this bird evidently had not decreased during the period of time since the last superinfection. The removal of the parasites from the other birds was considerably delayed and in some an increase in number was recorded. The control bird (29) which had no superinfections showed an increase of W parasites typical of this strain when inoculated into birds with latent N infections.

TABLE 7.—*Results of Injecting Strain W into Birds Previously Injected Repeatedly with Strain N*

Bird No.	No. of Superinj.	Test Strain	Parasites per 10,000 Red Cells								
			Days after injection								
			#	1	2	3	4	5	6	7	8
30	7	W	295	128	114	262	180	106	135	66	0
31	8	W	335	+		0			0		0
32	7	W	48	0	0	0	0				
33	8	W	620	25	†	0	0				
34	7	W	386	78	35	51	†	0		0	
42	0	W	208	425	256	87	32	†	†	0	0

10 minutes after injection.
† Estimated number of parasites 10 or less per 10,000 red cells.
+ Estimated number of parasites 10 to 35 per 10,000 red cells.

Five other birds were superinfected similarly to the first group except that the N instead of the R strain was used. The results are recorded in table 7. The final tests were made on these birds on or before the ninth day following the last superinfection. With one exception (bird 30) the immunity to strain W was very effective. Control bird 42 showed a typical example of the partial immunity produced by strain N to inoculations of strain W. Numerous superinfections of strain N apparently increase the partial cross-immunity to strain W to a degree comparable to the immunity to superinfection with homologous strains. After the seventh superinfection of birds 30, 31, and 34 examinations

were made 10 minutes after the injections to determine the relative number of parasites inoculated. These counts are representative of all the injections made as all the birds used for blood were heavily infected. Seven tenths to 0.8 cc. of blood-saline mixture were injected each time. It seems apparent from counts made that there were more than 100 parasites per 10,000 red blood cells in the superinfected birds after each injection.

TABLE 8.—*Results of Injecting Strain M into Birds Previously Injected Repeatedly with Strain N and Tested with Strain W*

Bird No.	No. of Superinj.	Test Strain	Parasites per 10,000 Red Cells								
			Days after injection								
			#	1	2	3	4	5	6	7	8
32	7(8)*	M	450	†	0	0					
33	8(9)*	M	290	21	0	0					
34	7(8)*	M	390	210	117	+	†				
35	0	M	480	526	1850	648	1022	2550	1443	662	143

10 minutes after injection.
* Plus the test (W) injection of Table 7.
† Estimated number of parasites 10 or less per 10,000 red cells.
+ Estimated number of parasites 10 to 35 per 10,000 red cells.

The immunity of birds 32, 33, and 34 was further tested by inoculating them with strain M. The results, (table 8), demonstrate that a very effective immunity was produced against this virulent strain also. The control bird 35 was carrying a latent infection of strain W. A single initial infection of strain W produces only a partial immunity to strain M. Comparable results were obtained when strain A was inoculated into birds carrying latent infections of strain W (table 3).

DISCUSSION

In the preceding experiments a strain of P. cathemerium and a strain of P. relictum have been studied which are morphologically identical with the respective classical strains, but are dissimilar in other characteristics. Although Huff[9] described strain R and noted its relatively short period of infection and lack of virulence, no experimental studies have been made previously on its immune relationship with other strains. Strain A has not been described previously.

If the term "virulence" as applied to these infections is restricted to the height of the infection at the crisis and the length of the acute infection, together with any other factors that may adversely affect the host, a correlation exists between the virulence of the strains and the relative immunity produced by them. In an ascending order of virulence the strains may be listed as follows: R, N, W, and A or M, the relationship shown by figure 1. Infections with strain R produce only slight symptoms which disappear in one or two days. Symptoms produced by infections with the N or W strains are similar, except that they last 5 or 6 days longer in the case of the latter. The A and M strains are the most virulent of the group, producing a mortality of 70%.

The same order of relationships exists in the immunity produced as measured by cross-infection. The immunity produced by strain R is only partially

effective against cross infections of any of the other strains (table 2). Strain N produces an immunity that is highly effective against strain R (table 1), but only partially protective against strains W and A. The results of inoculations of the various strains in birds carrying latent infections of strain W show that this strain produces an immunity that is highly effective in removing parasites of strains R and N, but is less effective against strain A. Because of a lack of birds carrying latent infections of strain A only the W strain was inoculated into birds with this strain, but these cross infections demonstrate that complete immunity was produced by strain A to strain W. To summarize these results, strain R, which is the least virulent, produces the least effective cross-immunity to the other strains. In contrast, strain A, the most virulent of the group, produces complete immunity to all the other strains used, but conversely none of the other strains show complete protection against strain A. The very effective immunity produced by strain W against cross infections of strain N holds for the less virulent strain R but not for the more virulent strain A. In like manner, the immunity produced by strain N is completely effective against the relatively avirulent strain R, but only partially effective in preventing cross infections with the more virulent strains W and A. The effect of the immunity produced by strain R on the removal of the parasites of the other strains when superinfected, appears to be correlated with the virulence rather than the species of the superinfected strains. When injected into birds with latent R-infections strain N is removed more readily than strain W (table 2), while strain W produces lower superinfections than the virulent strain A (birds 13 to 15, table 2, and birds 19 to 21, table 3). Though the cross-immunity among these strains shows them to be very closely related, a difference is noted in the varying susceptibility of the different strains to the developing immunity. In infections of strain R it becomes effective on the third day. Against infections of strain N it becomes effective about the sixth day, and of strain W about the eighth day after the appearance of parasites in the blood. Strain A is virulent enough for the infection to reach a point that generally results in the death of the bird before the immunity becomes effective.

In presenting a quantitative basis for the immune relationship of the various strains of P. cathemerium and P. relictum used, it is not intended that this is an explanation of group immune reactions in general. The conditions obtaining in the cross-immunity of the strains of malaria used in these experiments have appeared not to be easily explainable on the basis of group reactions that hold for other micro-organisms. The cross-immune reactions of these malarial strains differ in that the reciprocal crosses are not always comparable. Birds carrying latent infections of P. relictum possess a complete immunity to inoculations of P. cathemerium (Gingrich[6]), but the reciprocal cross is only partial. Also, infections of P. rouxi produce partial immunity to P. cathemerium and vice versa, but there is no cross-immunity between P. relictum and P. rouxi, (Gingrich). While the factor or factors responsible for the more or less definite limitation of each strain are unknown, one might say that one such factor is an equilibrium between the pathogenicity of the parasites and the immune reactions produced by the host. The question of why

such an established equilibrium varies with the various strains can only be answered by saying that it is a manifestation of the difference of the strains.

In contrast to the results found by Gingrich in which 5 strains of P. relictum isolated from widely separated geographical locations were found to be immunologically identical, strain R of P. relictum not only is ineffective in producing an immunity comparable to that of strain W, but also is ineffective against P. cathemerium. Furthermore, latent infections of P. cathemerium are highly protective against strain R. With the exception of the cross-immunity tests between the D strain and the atypical strain derived from it by Huff and Gambrell[11] it seems that the only strain of P. cathemerium which has been used in immunity tests is the original H strain isolated by Hartman.[1] The new strain designated as strain A of P. cathemerium which was isolated from a grackle and used in further cross-immunity tests in the present experiments also varies in its immune reactions from the classical cross-immune reactions between P. cathemerium and P. relictum. Cross-immunity studies between strain A of P. cathemerium and strain W of P. relictum show that latent infections of strain W produce only partial cross-immunity to strain A (table 3). Birds carrying latent infections of strain A (table 4) could effectively remove parasites of strain W.

The high degree of correlation between the immunity produced by a strain and its virulence indicates that a quantitative factor is involved in the development of the strain immunities. An antigenic analysis of the immunity to cross infections indicates that the various degrees of immunity produced by these strains depend on a single antigenic complex, the variable factor being the degree of stimulation of each infection. The more virulent strains may be presumed to have more stimulating effects because there are more parasites present in the initial infection. The complete immunity to cross infections produced by the more virulent strains against any less virulent strain probably results from a greater stimulation of the same mechanism by antigenic components common to each. The immunity to the less virulent strains is not completely effective in removing superinoculated parasites of more virulent strains, but the difference appears to be one of quantity only.

The demonstration that the immunity which is produced by strain N against strain W can be built up by multiple superinfections of the former further establishes the relationship of the immune reactions of the two strains. The partial immunity resulting from a single initial infection of strain N is slightly less effective against strain W than that produced by strain W itself. This difference correlates with the measures of virulence of these two strains, strain W being about twice as virulent as strain N. By virtually increasing the effective virulence of strain N infection by repeatedly injecting the bird with numerous parasites of the homologous strain, the effectiveness of the immunity against strain W is likewise increased. Increasing the number of parasites by superinfections has the same effect as a longer period of infection, with possibly a higher peak. Other methods of increasing the length of the infection or the number of parasites such as

11. Am. J. Hyg. **19:** 404, 1934.

blockade of the macrophages, or otherwise lowering the resistance of the bird might possibly interfere with the stimulation of the immune mechanism and hence not result in an increase in the resistance of the host. The increased number of parasites, in order to be effective, must be able to further stimulate the macrophages. The enhanced resistance produced by multiple superinfections of strain N against strain W leaves little doubt that the same antigenic factor is effective for both strains. The same conditions have been shown to apply to strain M.

The quantitative difference between the immune reactions produced by strains R and N is so much greater than that between strains N and W that many times the number of superinfections would be required in the former than in the latter case to build up the immunity. Another factor limiting the effectiveness of the R superinfections made was the fact that so few parasites were present in the inoculated blood. The relatively short and low initial infection of strain R is apparently capable of stimulating only a relatively few macrophages to activity, or stimulating those to a less degree but it is sufficient, however, to effect the removal of the parasites of this avirulent strain.

In this series of experiments one instance was found where the quantitative basis of the immune reactions does not entirely hold. Parasites of strain A inoculated into birds carrying latent infections of strain N are removed more rapidly than when inoculated into birds carrying latent infections of strain W (table 3). If a purely quantitative factor were involved the immunity produced by strain W would be more effective against cross infections of strain A than that produced by strain N. In all other instances of cross infections of the 4 heterologous strains used the most virulent has produced the best immunity. Parasites of strain W are effectively removed by birds with latent infections of strain A, but not by strain N. This latter fact demonstrates that a quantitative factor based on virulence is active. The apparently specific reactions between strains N and A is not specific in the sense of having no relationship with strain W. Apparently there is a common reaction between them not involved in the immune mechanism of strain W.

It is readily recognized that such superinfections as those carried on in these experiments are somewhat unnatural. All transfers of parasites have been made by blood inoculations of the asexual stage. However, the fact that from time to time each of the strains used has been passed through susceptible mosquitoes with the result that no noticeable variations have occurred signifies that no modification of the physiology has been effected. The A strain has been kept latent in canaries almost from the time it was isolated until the present work was begun in the spring of 1936. Later in the same year this strain was established in mosquitoes which transmitted it to canaries. These infections were similar in every respect to infections previous to the mosquito transfer. Likewise, Huff has transferred strain R by mosquito passage and noticed no change in its morphology or physiology. Very little can be said about the occurrence of strains R and A in nature. Strains A and M though isolated from two different hosts appear to be identical. No other report of a similar strain has been found. Wolfson[12] isolated a strain of P. relictum which

12. Am. J. Hyg. **25**: 177, 1937.

would seem to be identical with strain R except that it is more virulent. No further record of a comparable strain has been found.

The results obtained by Mulligan and Sinton[13] with multiple superinfections of P. knowlesi, in Silenus rhesus, do not exactly correspond to the results obtained in the present experiments. The various strains of P. knowlesi used by these workers were isolated from different specimens of malayan monkeys, Silenus irus. The primary characteristic by which strains were differentiated was a slight clinical variation. When cross infections were made, some strains, generally the more virulent ones, were found to produce a more effective tolerance than other strains. No cross-immunity of species was found between P. knowlesi and P. inui var. cynomolgi. According to Mulligan and Sinton all strains of P. knowlesi are very virulent for S. rhesus, while infections in S. irus are very mild. In S. rhesus the tendency to relapse is very great and a spontaneous recovery is seldom encountered. Quinine treatment is frequently necessary in superinfections and almost always required in the initial infection to save the life of the monkey. The infection of P. knowlesi in S. irus more nearly correspond with infections of most strains of avian malaria in canaries, especially as to virulence. Only strains A and M of avian malaria seem to be as virulent for canaries as P. knowlesi for S. rhesus. Similar experiments using strains A and M, and probably others of like virulence, might be expected to give results more nearly resembling those of Mulligan and Sinton. One outstanding feature of both groups is the fact that the most virulent strains produce the most effective immunity to other strains. In the avian malarial strains both the virulence and the immunity of various strains are sufficiently different to permit quantitative measurements of the relationships. In the simian malaria used by Mulligan and Sinton[13] there is a lack of sufficient variation among the strains to make possible a distinct separation either on immunological or pathogenic bases.

CONCLUSIONS

Strain R of P. relictum, var. matutinum, and strain A of P. cathemerium have been found to vary immunologically from the reactions of these two species as described by Gingrich. The virulence of strains R and W of P. relictum and strains N and A of P. cathemerium correlates with the immune reactions of the same strains. Strain R is the least virulent and produces the least effective immunity; next in the series is strain N which is lightly less virulent than strain W, the latter producing complete immunity to the 2 previous strains, and finally strain A is the most virulent and produces the most effective immunity. Birds carrying latent infections of strain A are completely immune to inoculations of strain W of P. relictum. This quantitative relationship is further demonstrated by the effective increase in the immunity of strain N to strain W by multiple superinfections of the former.

13. Records of the Malarial Survey of India **3:** 809, 1933.